Bilirubin and glutathione have complementary antioxidant and cytoprotective roles

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Glutathione (GSH) and bilirubin are prominent endogenous antioxidant cytoprotectants. Despite tissue levels that are thousands of times lower than GSH, bilirubin is effective because of the biosynthetic cycle wherein it is generated from biliverdin by biliverdin reductase (BVR). When bilirubin acts as an antioxidant, it is oxidized to biliverdin, which is immediately reduced by BVR to bilirubin. Why does the body employ both of these 2 distinct antioxidant systems? We show that the water-soluble GSH primarily protects water soluble proteins, whereas the lipophilic bilirubin protects lipids from oxidation. Mice with deletion of heme oxygenase-2, which generates biliverdin, display greater lipid than protein oxidation, while the reverse holds for GSH depletion. RNA interference depletion of BVR increases oxidation of lipids more than protein. Depletion of BVR or GSH augments cell death in an oxidant-specific fashion.

apoptosis | biliverdin | cell death | heme oxygenase | neuroprotection

Bilirubin is the end product of heme metabolism. Free heme, which is toxic, is degraded via cleavage of its tetrapyrrolole ring by heme oxygenase (HO) (1). Two major forms of HO exist. H01 is an inducible enzyme that occurs in many tissues but is most abundant in the spleen where it is activated by heme emerging from degraded red blood cells. H01 is rapidly induced by diverse cytotoxic stimuli and is regarded as one of the heat shock proteins. By contrast, HO2 is constitutive and most concentrated in brain and testes. In the brain and peripheral nervous system carbon monoxide, formed when the heme ring is cleaved, appears to be a neurotransmitter (2, 3). Its synthesis is regulated by neuronal activity, as depolarization of neurons leads to calcium entry with calcium-calmodulin binding to and activating HO2 (4). Besides forming CO, opening of the heme ring generates the linear tetrapyrrolole biliverdin. Biliverdin accumulates very little in most tissues being rapidly reduced to bilirubin by the high tissue densities of biliverdin reductase (BVR) (5).

Because bilirubin is toxic and insoluble, it must be glucuronidated before excretion in the bile. The glucuronidation pathway is poorly developed in most newborns leading to accumulation of bilirubin whose yellow color conveys the physiologic jaundice of many babies. Substantial elevations of bilirubin lead to its deposition in the brain with kernicteric damage.

Because biliverdin is more water soluble than bilirubin, hence more readily excreted, the physiologic rationale for the existence of the BVR pathway has been unclear. Bilirubin is a potent antioxidant that may provide cytoprotection (6, 7). However, tissue concentrations of bilirubin, about 20−50 nM, are much too low to cope with the mM levels of reactive oxygen species that most cells encounter. By contrast, GSH, a well-accepted physiologic cytoprotectant antioxidant, occurs in levels of 5–10 mM in most tissues.

We provided evidence that bilirubin can be a physiologic antioxidant neuroprotectant (8). Mice with targeted deletion of HO2 ($HO2^{-/-}$) have reduced bilirubin levels and are more susceptible to neurotoxic damage, seizures, stroke damage, and traumatic brain injury (9–12). The cytotoxicity associated with deletion of HO2 appears to reflect a loss of bilirubin. Thus, as little as 10 nM bilirubin protects against 10,000 higherfold concentrations of hydrogen peroxide H_20_2 (8). The antioxidant actions of bilirubin are dramatically amplified by BVR in a biliverdin–bilirubin cycle (13). Thus, when bilirubin acts as an antioxidant, it is itself oxidized to biliverdin which is rapidly reduced by BVR to bilirubin. Depletion of BVR by RNA interference markedly diminishes the cytoprotective effects of exogenous bilirubin and leads to increased cellular levels of oxygen free radicals and cell death (13). The augmentation of cytotoxicity following BVR depletion is greater than that following depletion of GSH by buthionine sulfoximine (BSO), an inhibitor of γ-glutamylcysteine synthase, the rate limiting enzyme in GSH synthesis.

We wondered why cells have evolved 2 distinct antioxidant cytoprotectant systems, bilirubin and GSH. Bilirubin is highly lipophilic, while GSH is hydrophilic. Conceivably bilirubin protects against lipid peroxidation of cell membranes while GSH largely protects water soluble proteins. In the present study we demonstrate a selective augmentation of lipid peroxidation associated with bilirubin depletion while GSH depletion is associated more with augmented oxidation of water soluble proteins.

Results

To explore the importance of the HO2/BVR systems in protecting differentially lipid and protein compartments of cells from oxidative stress, we used mice with targeted deletion of HO2 (Fig. 1*A*). In the brains of HO2-deleted mice, we detect a major augmentation in levels of 4-hydroxynonenal (4-HNE). Thus, under these basal conditions, physiologic levels of HO2 and its metabolic products appear to be protecting against lipid peroxidation. We monitored the oxidative status of proteins by assaying protein carbonyls. Levels are unaltered in HO2 mutants implying that H02 and its products do not afford major protection against protein oxidation (Fig. 1 *B* and *C*). To ensure that our carbonyl assays reflect the effects of oxidative stress on protein oxidative status, we treated brain and liver lysates with tert-butyl hydroperoxide (TBH), which triples protein carbonyl levels in brain and liver with no difference between wild-type and HO2-deleted mice.

Our earlier studies indicated that bilirubin is the metabolic product of the HO2 system that mediates antioxidant effects (8). Accordingly, we directly examined antioxidant effects of bilirubin and compared these to actions of GSH. In HEK293 cells,

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The authors declare no conflict of interest.

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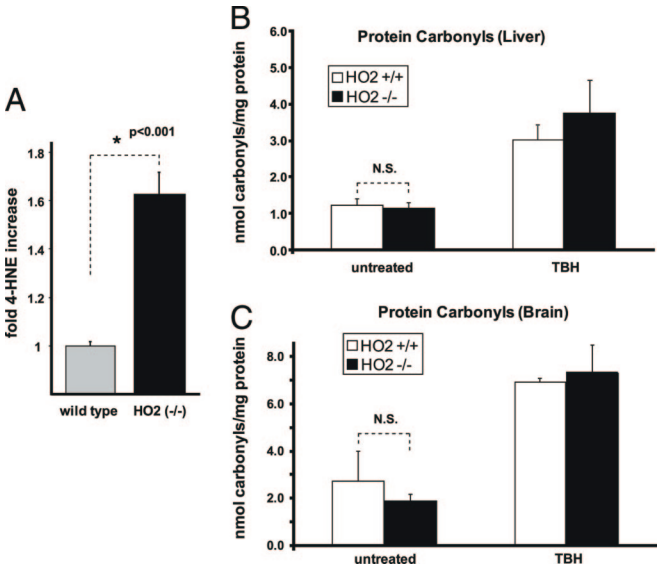

Fig. 1. Increased lipid but not protein oxidation in heme oxygenase-2 knockout (HO2 $^{-/-}$) animals. (*A*) Increased lipid oxidation product 4-hydroxynonenal (4-HNE) in HO2 (−/−) hippocampus. Data represent mean ± SEM from 4 samples. *, $P < 0.001$ by *t* test. (*B* and *C*) Similar levels of protein carbonyls, a measure of protein oxidation, in wild-type and HO2 (−/−) animals. Liver (*B*) and brain (*C*) were homogenized from 6-month-old male wild-type and HO2 (−/−) animals and protein carbonyls were determined by derivatization with 2,4-dinitrophenylhydrazine (DNPH). To verify measurement of increased carbonyls, samples were treated 30 min with 1 mM tert-butyl hydroperoxide (TBH). Means ± SEM are shown for 4 animals from 2 independent studies.

lipid peroxidation, monitored by 4-HNE levels, is greatly increased by TBH treatment with the increase abolished by 100 μM bilirubin (Fig. 2*A*). We compared effects of bilirubin and the water soluble antioxidants ascorbate and GSH upon protein oxidation monitored in terms of levels of oxidized methionine (Fig. 2*B*). While hydrogen peroxide preferentially oxidizes lipids, it also leads to protein oxidation. The 6-fold increase in

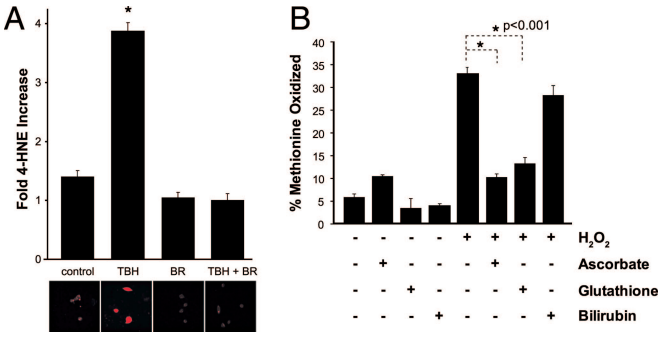

Fig. 2. Differential protection of lipid and protein oxidation by bilirubin and glutathione. (*A*) Bilirubin blocks oxidant-induced accumulation of 4-hydroxynonenal (4-HNE) adducts. HEK293 cells were treated with combinations of 500 μM tert-butyl hydroperoxide (TBH) and 100 nM bilirubin (BR) for 16 h and stained for 4-HNE. Data represent means ± SEM from 4 samples. *, $P <$ 0.001 from each other treatment (ANOVA followed by Tukey-Kramer post hoc test). Representative of 3 experiments. (*B*) Greater potency of water-soluble antioxidants for protein oxidation than bilirubin. HEK293 cells were radiolabeled with [^{35}S]methionine, and protein was oxidized with combinations of H_2O_2 (10 μM), bilirubin (5 μM), and the water-soluble antioxidants, glutathione (5 μM) and ascorbate (5 μM). Data represent means ± SEM of triplicate determinations, representative of 3 experiments. *, $P < 0.001$ (ANOVA followed by Tukey-Kramer post hoc test).

protein oxidation elicited by hydrogen peroxide is almost completely reversed by ascorbate and GSH but not by bilirubin, consistent with selective protection of lipids by bilirubin.

To ascertain the importance of endogenous bilirubin in cytoprotection, we designed vectors for RNA interference (Fig. 3*A*). Two of these, 180 and 728, almost totally deplete BVR in HEK293 cells (Fig. 3*A*). The BVR depletion is selective with no changes in H01, H02, α-tubulin, IP3 receptors, IP6 kinase-2, Bcl-x, or Bax (supporting information (SI) Fig. S1). BSO, a specific inhibitor of γ-glutamylcysteine synthase, reduces basal GSH levels by 96% within 24 h (Fig. S2). In basal, untreated HEK293 cells with BVR depletion, endogenous levels of 4-HNE are increased 2.5- to 3-fold to levels resembling those elicited by hydrogen peroxide (Fig. 3*B*). By contrast, BSO treatment elicits only a 30% increase in 4-HNE. These findings support the notion that endogenous BVR generating bilirubin plays a substantially greater role in protecting against lipid peroxidation than does endogenous GSH.

We wondered whether the protective functions of endogenous bilirubin and GSH selectively impact oxidant-induced cell death. Accordingly, we depleted BVR using 2 different siRNA constructs 180 and 728 and ascertained the influence of oxidants on cell death. In HEK293 cells depletion of BVR markedly increases the cytotoxic actions of TBH or hydrogen peroxide (Fig. 4 *A* and *C*). In HeLa cells cytotoxic effects of hydrogen peroxide are also exacerbated by BVR depletion (Fig. 4*B*).

In BVR depleted cells, overexpression of an siRNA-resistant BVR restores levels of BVR (Fig. S3*A*). This procedure partially restores viability of cells treated with hydrogen peroxide (Fig. S3*B*). Cell death associated with BVR depletion appears to be largely apoptotic, as it is associated with poly(ADP-ribose) polymerase (PARP) cleavage (Fig. S4*A*) and caspase activity (Fig. S4*B*).

In the brain bilirubin also is cytoprotective. Thus, in cerebral cortical cultures depletion of BVR by 2 distinct siRNA constructs produces an 85–90% decrease in cell viability (Fig. 4*D*). A similar increase in cytotoxicity associated with BVR depletion is evident in the hippocampus although the effect is somewhat less (Fig. 4*E*).

The differential effects of bilirubin and GSH on lipid and protein oxidation predicts that the 2 agents should be selectively cytoprotective for lipophilic and hydrophilic insults. While 6-hydroxydopamine promotes lipid and protein oxidation but with greater effects upon protein oxidation (14), hydrogen peroxide evokes greater lipid oxidation than protein oxidation unless exogenous iron is supplied (15, 16). We hypothesized that these 2 oxidative stressors would respond differentially to depletion of GSH and BVR.

We detect no increase in cell death with BVR or GSH depletion in untreated cells. Depletion of GSH by BSO treatment fails to augment cytotoxic actions of hydrogen peroxide, while BVR depletion leads to a 4-fold increase in cell death (Fig. 5*A*). Treating BVR depleted cells with BSO provides a further increase in cellular toxicity. By contrast, in 6-hydroxydopamine-treated cells BSO provokes a 6-fold increase in cytotoxicity, almost twice the increase elicited by BVR depletion (Fig. 5*B*). Cytotoxicity is further augmented by the combination of BVR and GSH depletion. These findings further support the notion that BVR, via bilirubin, selectively protects cells from oxidative assaults on the lipids of cell membranes whereas GSH selectively protects water soluble proteins.

Discussion

The principal finding of this study is that bilirubin and GSH pathways provide physiologic cytoprotection of a complementary nature. Bilirubin largely protects against lipid peroxidation while GSH primarily prevents the oxidation of water soluble proteins. Nonetheless, the 2 systems do possess overlapping

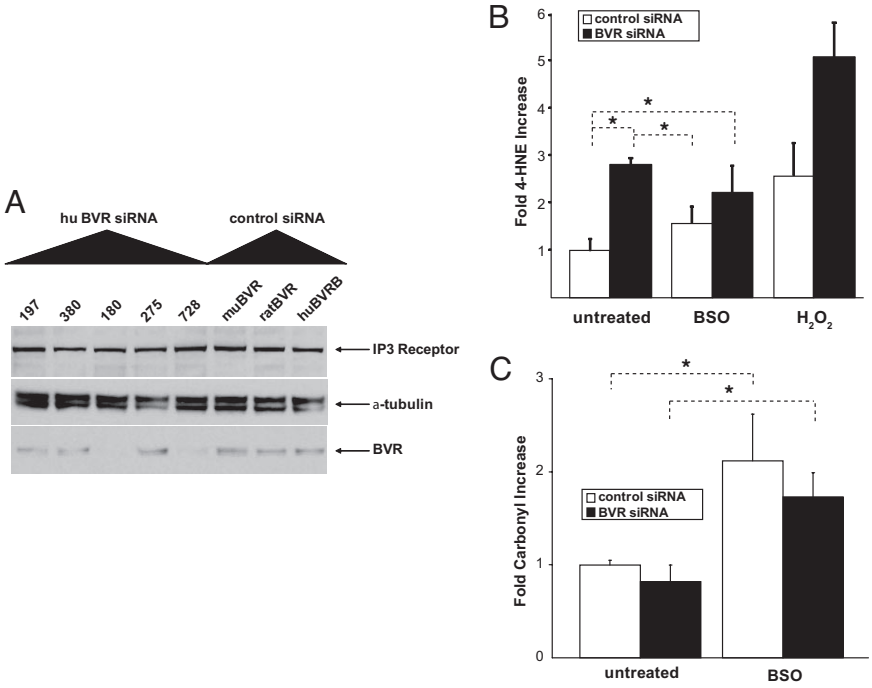

Fig. 3. Depletion of BVR preferentially increases lipid oxidation while GSH depletion increases protein oxidation. (*A*) siRNA depletion of biliverdin reductase (BVR). HEK293 cells were transfected with the indicated siRNA and lysates immunoblotted at 72 h. siRNA 180 and 728 had the greatest efficacy in depleting BVR. Representative of 3 experiments. (*B* and *C*) Greater lipid oxidation follows depletion of biliverdin reductase (BVR) than glutathione. HEK293 cells were transfected with BVR siRNA (180) or control siRNA (muBVR), and then treated with 1 mM buthionine sulfoximine (BSO) at 48 h to rapidly deplete glutathione. At 72 h posttransfection, cells were treated with 250 μM H_2O_2 for 16 h and stained for lipid oxidation product, 4-hydroxynonenal (4-HNE). In *C*, protein carbonyls were quantified by derivatization with 2,4-dinitrophenylhydrazine (DNPH). Data represent means ± SEM of quadruplicate determinations. *, $P < 0.01$ (ANOVA followed by Tukey-Kramer post hoc test) (*B*); *, $P < 0.05$ (*t* test) (*C*).

functions. Thus, GSH can protect against lipid oxidation, and some GST A1–1 isoenzymes influence lipid hydroperoxides (17). We find greater potency of bilirubin in preventing oxidative degradation of lipids, while GSH preferentially prevents oxidation of water soluble proteins. Membrane proteins exist in a hydrophobic environment where bilirubin might be more important than glutathione for maintaining their stability. The microenvironment may largely determine whether bilirubin or GSH predominates for antioxidant activity. Thus, glutathione may be primarily responsible for protecting diverse substances that are cytosolic while bilirubin plays a similar role in membranes.

The complementary functions of bilirubin and GSH are physiologically relevant, as depletion of bilirubin by HO2 or BVR deletion both selectively enhance lipid peroxidation. On the other hand, depletion of GSH by BSO, which inhibits γ-glutamylcysteine synthase, the rate limiting enzyme in GSH formation, selectively augments water soluble protein oxidation. These antioxidant effects impact cell survival, as cell death is more markedly augmented following depletion of bilirubin or GSH depending upon the type of oxidant.

All cells in the body are exposed to a dynamically changing environment of reactive oxygen species. It is thought that multiple substances provide physiologic antioxidant influences. Besides bilirubin and GSH, uric acid, ascorbate, vitamins A and E, ergothioneine, and possibly melatonin are physiologic antioxidants. It is difficult to assess the relative importance of these various substances. Some are formed endogenously, such as bilirubin, melatonin, GSH, and uric acid, while others are exogenous, such as vitamins A and E, ascorbate, and ergothioneine. Some are largely lipophilic, such as bilirubin, vitamins E and A, while GSH, ascorbate, uric acid, and ergothioneine are more water soluble.

One approach to considering the relative importance of these substances would be to compare their endogenous concentrations. Circulating and tissue GSH levels range from 1 to 10 mM, probably the highest of the physiologic antioxidants. The approximate circulating concentrations of other substances are 30–100 μM ascorbate (vitamin C), 0.3–0.6 μM beta-carotene, 2–28 μM alpha-tocopherol (vitamin E), 1 mM ergothioneine, 0.2–0.4 mM uric acid, 50 pmol/L melatonin, and 5–15 μM bilirubin, with tissue concentrations significantly lower (18). Such comparisons can be misleading. Thus, antioxidant actions of bilirubin may be amplified 10,000 times or greater by the BVR cycle (13). GSH undergoes some cycling with GSH peroxidase oxidizing GSH to GS-SG, after which GSH reductase recycles it back to GSH. The manyfold increase in lipid and protein oxidation elicited respectively by BVR and GSH depletion implies that these 2 substances are major physiologic antioxidants. The importance of bilirubin in cytoprotection is indicated by the 60–80% decrease in hippocampal/cortical neuronal viability associated with BVR depletion.

Ways in which the various antioxidants interact are not clear. There could be back-up systems whereby reactive oxygen species would be attacked by 1 antioxidant as a "first line of defense," with escaping molecules handled by a second collection of antioxidants. Alternatively, the various antioxidants may deal with different classes of reactive oxygen species. For bilirubin and GSH, our findings favor the latter hypothesis.

The concept of discrete lipophilic and hydrophilic domains of cytoprotection has clinical implications. Because of the importance of oxidative stress in many diseases, clinical trials of antioxidants have been pursued for cardiovascular disease, cancer, and dementia. Extensive studies with vitamin E have largely been disappointing (19–23). Indeed, a meta-analysis of many such studies by Miller and associates (24) suggests increased

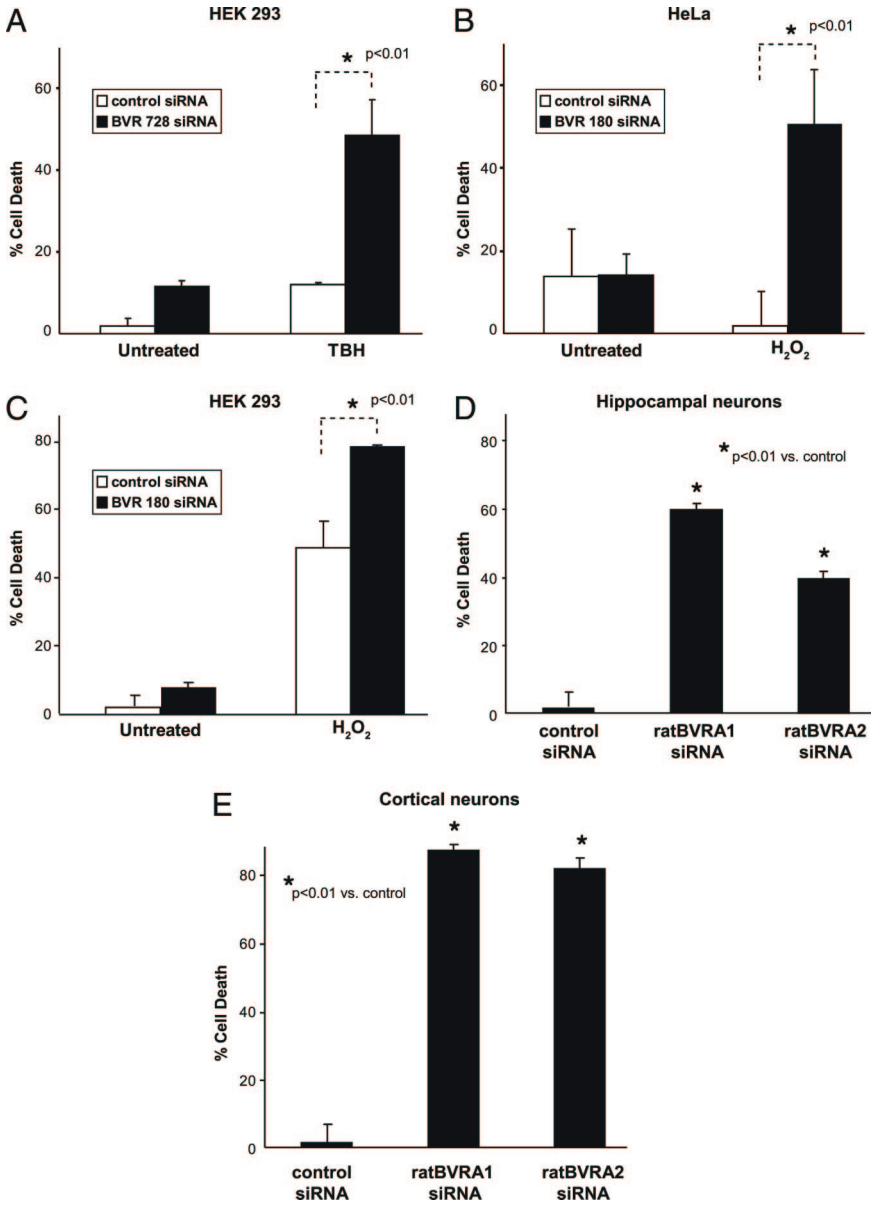

Fig. 4. Increased oxidant-induced cell death following depletion of BVR. (*A–C*) Increased susceptibility to oxidant-induced death in HEK293 and HeLa cells depleted of BVR by 180 and 728 siRNA. HEK293 cells were treated with the indicated siRNA for 72 h then exposed to hydrogen peroxide (H_2O_2) or tert-butyl hydroperoxide (TBH) for 18 h. Cell death was assayed by MTT assay. Both the 180 and 728 siRNA elicited increased oxidant-induced cell death. (*A*) HEK 293 cells treated with 728 siRNA and TBH. (*B*) HeLa cells treated with 180 siRNA and H_2O_2. (*C*) HEK 293 cells treated with 180 siRNA and H_2O_2. Data are the means of triplicates ± SEM, representative of 2 experiments for each. *, $P < 0.05$ by *t* test. Control oligonucleotides: (*A* and *B*) muBVR, (*C*) BVR180c. (*D* and *E*) Neuroprotective effect of BVR in primary hippocampal (*D*) and cortical (*E*) neurons. Two different siRNAs targeting endogenous rat BVR (ratBVR1 and ratBVR2) or control siRNA directed against mouse BVR were cotransfected with eGFP. At 72 h posttransfection, surviving GFP positive neurons were blindly counted under 20× magnification in 10 fields. Data are representative of 4 independent transfections. *, $P < 0.01$ vs. control (ANOVA followed by Tukey-Kramer post hoc test).

mortality in those with high-dose vitamin E supplementation. If bilirubin is more important than vitamin E in providing cytoprotection, treatments that augment endogenous bilirubin levels might be more effective. Because bilirubin has been traditionally thought to be a toxic end product of heme metabolism with no therapeutic relevance, pharmacologic tools to alter its levels have not been heretofore developed. Agents that block the binding of bilirubin to plasma proteins might enhance levels of free bilirubin that could enter cells. Alternatively, selective inhibitors of bilirubin glucuronidation may be useful. The disappointing clinical results with lipophilic vitamins may stem from the failure to simultaneously address oxidative damage of water soluble proteins. Perhaps combination treatments of hydrophilic and lipophilic antioxidants would be more effective.

Plasma levels of bilirubin have been extensively measured in various clinical conditions. While very high levels of bilirubin are certainly neurotoxic, numerous investigations indicate that mildly elevated levels of bilirubin can be beneficial (6, 7). For instance, elevated serum levels of bilirubin are associated with diminished risk of coronary artery disease (25). In one case-control study, patients with a familial history of coronary artery disease displayed lower serum bilirubin levels than those without such a history (26). The protection evidently afforded by bilirubin in this investigation was similar to that associated with high-density lipoprotein cholesterol. Gilbert syndrome is a common genetic condition involving impairment of bilirubin conjugation leading to elevated serum bilirubin levels (27). The

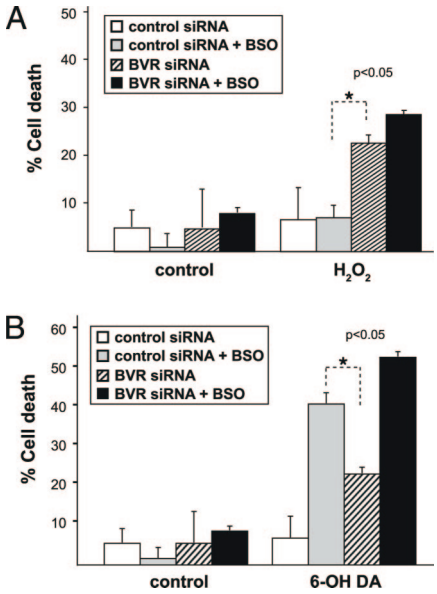

Fig. 5. BVR and glutathione (GSH) pathways, respectively, protect against oxidants that differentially target lipids and protein. (*A*) Depletion of BVR enhances hydrogen peroxide (H_2O_2)-induced cell death greater than glutathione depletion. HEK293 cells were transfected with the BVR or control siRNA, then treated with 1 mM BSO at 48 h to deplete glutathione. At 72 h posttransfection, cells were treated for 14 h with 500 μM H_2O_2 and cell death was determined by MTT assay. BVR depleted cells exhibit markedly increased cell death compared to GSH-depleted cells. (*B*) Greater protection against 6-hydroxydopamine (6-OH DA)-induced cell death is provided by the GSH pathway than the bilirubin pathway. Cells were depleted of BVR and GSH as in *A*, then treated 14 h with 300 μM 6-OH DA. Cell death is greater following depletion of GSH than BVR. Data are means of triplicates from at least 3 independent experiments. *, $P < 0.05$ by *t* test.

prevalence of ischemic heart disease in individuals with Gilbert syndrome is 2%, about one-sixth that of a control population (28). In this study elevated bilirubin levels provided a better index of disease protection than high-density lipoprotein cholesterol. In a meta-analysis of 11 studies, the risk of atherosclerosis was diminished in individuals with elevated bilirubin levels (29). The risk of carotid plaques was reduced by about a third in individuals with elevated bilirubin levels (30).

The HO/BVR pathway has important roles other than its antioxidant activities. Carbon monoxide, a product of the HO reaction, can act as a neurotransmitter (2, 3) and anti-inflammatory/antiproliferative molecule (31). Bilirubin can inhibit oxidant associated-neutrophil chemotaxis (32). Maines and associates reported that BVR can act as a serine/threonine-tyrosine kinase (33, 34) and a transcription factor (35).

Methods

Depletion of Cellular Biliverdin Reductase (BVR). All reagents were purchased from Sigma Chemicals (St. Louis, Missouri) except where noted. Antibodies were obtained from Affinity Bioreagents (BVR) and Santa Cruz (Bax, Bcl-x). Rabbit HO1, HO2, and IP3 receptor antiserum are previously described (4, 36). Monoclonal antibody HNEJ2 against 4-HNE was a gift of K. Uchida (Nagoya University, Japan).

To deplete BVR, 6×10^5 cells were seeded into each well of a 24-well dish and allowed to grow in complete media for at least 24 h until cells reached 80% confluence. Depletion of BVR was achieved via rational design of 19 base complementary siRNA oligonucleotides (Dharmacon). The oligonucleotides have 3′ dTdT overhangs to enhance stability with corresponding sense sequence as follows: human BVR180 GGAUGCUCUUUCCAGCCAA, human BVR728 AUGUAGGAGUGAAUAAGAA, human BVR197 AAGAGGUGGAGGUCGCCUAUA, human BVR380 AACUCUUGAUGGAGGAAUUCG, rat BVRA1 AGAUUGAUGUCGCCUAUAU, rat BVRA2 CAGCCAUGAAGACUAUAUA, mouse BVR231 UACCCCAUGGCAUUGUCAU, and human BVRB CAAGCAGGUUACGAAGUGA. BVR180c GGuUGCUCUaUCCAaCCAg is a control oligonucleotide for BVR180 with lowercase letters indicating 4 sites in which nucleotides were exchanged within the sequence itself (Fig. S1). Human and mouse BVR sequences are also sufficiently different such that mouse BVR oligonucleotides could be used as transfection controls for depletion of human BVR (Fig. 3). One microliter of lipofectamine 2000 (Invitrogen) was added to 49 μL of serum-free Optimem media (Invitrogen) and incubated for 5 min at room temperature. RNAi-fect (Qiagen) was also used as a transfection reagent at the same dosage. siRNA oligonucleotides (75 pmol/uL) were diluted in Optimem and mixed with the lipofectamine. Following a 20-min incubation, the complexes were added to the cells, which were in 500 μL of antibiotic-free media, achieving a final concentration of 33.3 nM siRNA. After 4 h the media were carefully removed and replaced with complete media. After 72 h the cells were analyzed or treated further with oxidants. Cells were also cotransfected with an enhanced green fluorescent protein-expressing plasmid (eGFP, Invitrogen) in an identical procedure using 100 ng plasmid and 10 pmol siRNA. Plasmids were purified with Qiagen Maxiprep columns to absorbance 260/280 ratios >1.8.

Depletion of Cellular GSH. Cells were treated with 1 mM BSO to deplete GSH. To quantify reduced (GSH) and oxidized (GSSG) glutathione levels following BSO treatment, we used a modification of the method of Baker et al. (37). Cells were cooled on ice, washed and scraped into buffer (1 mM EDTA, 100 mM sodium phosphate pH 7.5), and homogenized. Lysates were recovered by centrifuging 15 min at 10,000 $\times$ *g* to remove insoluble debris. Protein concentration was determined and equal volume of 0.1 g/mL metaphosphoric acid was added for 5 min and the sample centrifuged 2 min to recover supernatant. Triethanolamine was added to a final concentration of 2.65%. GSSG standards were prepared in the same buffer as serial dilutions. GSH and GSSG were developed colorimetrically by reactions containing a 50-μL sample and 100 μL of 0.15 mM 5,5′-Dithiobis(2-nitrobenzoic acid), 0.2 mM NADPH, and 1 unit/mL GSH reductase. Reactions were monitored at 405 nm and glutathione concentrations expressed as nmol/mg protein. To calculate GSSG, samples and a separate set of standards were pretreated 60 min with 10 mM 2-vinylpyridine, diluted from a 1 M stock in ethanol.

Analysis of 4-HNE. Reagents were obtained from Sigma, except where noted. Cells were grown in 2-well glass slide chambers (Lab-Tek) pretreated overnight with 0.25 mg/mL poly-D-lysine, washed 3 times with sterile water, then dried 2 h. After experimental manipulation, the plastic well was removed and the slide was gently washed 3 times in a well of PBS, then fixed 20 min with 3.7% paraformaldehyde/4% sucrose in PBS (made fresh, filtered). Slides were washed 3 times for 5 min in PBS with 10 mM glycine, permeabilized 15 min in 0.1% Triton X-100 in PBS/glycine, then washed 3 times in PBS/glycine. Slides were blocked in 5% normal serum 1 h, using the species for which the second antibody was derived. Blocking solution was removed and first antibody diluted in 0.5% BSA in PBS was added to the samples (1:3000–1:10000 for HNEJ2, 1:500 anti-activated caspase-3, Calbiochem). The HNEJ-2 antibody to 4-hydroxynoneal was a gift of K. Uchida, Nagoya University, Japan. Slides were incubated 1 h at room temperature, washed 3 times in PBS/glycine, and second antibody 1:300 diluted in 0.5% BSA in PBS/glycine (Alexa Fluor 488 or 568, Molecular Probes). After a 1-h incubation in the dark, slides were washed 3 times in PBS/glycine and nuclei stained with 1 μg/mL Hoechst 33342 (Molecular Probes) in PBS/glycine for 5 min. Slides were washed 3 times in PBS/glycine and coverslips mounted with Vectashield (Vector Labs) then sealed with nail polish. After drying 30 min, samples were visualized on a LSM 510 metaconfocal microscope (Zeiss) using excitation/emission wavelengths (nm) of 364/450 (Hoechst 33342), 488/525 (Alexa Fluor 388), and 568/600 (Alexa Fluor 568). Images were blindly acquired with LSM 510 software (Zeiss) with equalized background. A minimum of 3 fields per group were quantified with National Institutes of Health Image-J software for average signal intensity per unit of cell area.

Protein Oxidation. Protein carbonyls (38) were quantitated by derivatization with 2,4-dinitrophenylhydrazine (DNPH) and methionine oxidation determined by the method of Fliss and Brot (39). Tissue was homogenized in 10% wt/volume of 50 mM phosphate buffer, pH 7.4 and centrifuged 10,000 $\times$ *g* for 15 min to remove insoluble debris. The supernatant was divided into equal samples to which 4 volumes of 10 mM DNPH in 2 M HCl, using 4 volumes of 2 M HCl in the second tube for a blank. Samples were rotated 1 h at room temperature in the dark, precipitated with an equal volume of 20% trichloroacetic acid, placed on ice 10 min, and centrifuged at 10,000 $\times$ *g* for 5 min. Protein was resuspended in 1 mL 10% TCA, and reprecipitated. The pellet was washed 3 times with ethanol/ethyl acetate (1:1) to remove free DNPH and lipid contaminants, resuspending and centrifuging each time. Pellets were resuspended in 6 M guanidine HCl and centrifuged. Absorbance at 375 nm of 220

μL was obtained and carbonyls calculated as (A375 sample-A375 blank)/0.011 $\mu M^{-1} \times$ (resuspension/starting volume), 0.011 being the extinction coefficient. For siRNA experiments a smaller amount of protein was available and equal amounts of protein were derivatized 15 min with 10 mM DNPH and 3% SDS, followed by neutralization with 1.5 volumes of 2M Tris base/30% glycerol. Samples were loaded onto slot blots and immunoblotted with monoclonal anti-DNPH antibody.

Caspase and Cell Viability Assay. Caspase activity was detected via the EnzChek Caspase-3 Assay (Molecular Probes). Cells were placed in lysis buffer (150 mM NaCl, 1% Triton X-100, 50 mM Tris·HCl, pH 7.4, 1 mM EDTA), centrifuged at 16,000 $\times$ *g* for 10 min at 4 °C. Equal protein amounts were diluted in lysis buffer to 50 μL volume. Reactions were started by adding 50 μL of 2$\times$ reaction buffer (20 mM Pipes, pH 7.4, 4 mM EDTA, 0.2% Triton X-100, 10 mM DTT, and 50 μM Z-DEVD-R110) and monitored on a Perkin–Elmer LS55 Fluorimeter with excitation 496 nm and emission 520 nm. Cell viability was assessed with 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT). MTT was added to cell cultures at 125 μg/mL for 60 min. The media were carefully removed and the dye solubilized in dimethyl sulfoxide, using an empty well as a blank for the procedure. Absorbance was read at 560 nm, using 630 nm as a reference for debris. The debris and blank signal were subtracted and normalized to 100% for untreated samples.

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